MOLECULAR TECHNIQUES REVEAL WIDE PHYLETIC DIVERSITY OF HETEROTROPHIC MICROBES ASSOCIATED WITH *DISCODERMIA* SPP. (PORIFERA: DEMOSPONGIAE)

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Sponges are well known to harbor large numbers of heterotrophic microbes within their mesohyl. Studies to determine the diversity of these associated microbes have been attempted for only a few shallow water species. We cultured various microorganisms from several species of *Discodermia* collected from deep water using the 'Johnson-Sea-Link' manned submersibles, and characterised them by standard microbiological identification methods. Characterisation of a small proportion (ca. 10%) of the total and potential eubacterial isolate collection with molecular systematics techniques revealed a wide diversity of microbes. Phylogenetic analyses of 32 small subunit (SSU) 16S-like rRNA gene sequences from different microbes indicated high levels of taxonomic diversity associated with this genus of sponge. For example, bacteria from at least five eubacterial subdivisions — gamma, alpha, beta, *Cytophaga* and Gram positive — were isolated from the mesohyl of *Discodermia*. Several strains were unidentifiable from current sequence databases. No overlap was found between sequences of 24 isolates and 8 sequences obtained by PCR and cloning directly from sponge samples. The abundance and diversity of microbes associated with sponges such as *Discodermia* suggest that they may play important roles in marine microbial ecology, dispersal and evolution. ❐ *Porifera, Discodermia, microbial diversity, bacterial symbionts, in vitro culture, gene sequencing, 16S rRNA.*

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Many marine sponges harbor numerous microbial symbionts or 'associates'. Researchers enumerating and characterising sponge-microbial interactions have shown that bacterial biomass can reach over 50% in some marine sponges with corresponding phenomenally huge diversity (Vacelet & Donaday, 1977; Simpson, 1984; Wilkinson, 1987; Santavy et al., 1985; Fuerst et al., 1999, this volume). Although studies of sponge-bacterial associations are hampered by upper microorganism culturability limits, estimated to be below 1-10% (Austin, 1988; Eguchi & Ishida, 1990; Button et al., 1993), focus on marine microbial ecology and taxonomy is increasing. For example, novel aspects of diverse microbes and their habitats are being revealed by molecular genetics approaches (Amann et al., 1995; McInerney et al., 1995; Distel et al., 1988; Delong, 1998; R. Hill pers.comm.).

An understanding of the biology of marine sponges in the polyphyletic order 'Lithistida' would be enhanced by characterisation of the constituent organisms coexisting with these sponges, e.g. species richness, ecological function, metabolic load, etc. Lithistids in general, and *Discodermia* in particular, are the source of several compounds with pharmaceutical potential (Longley et al., 1991; Kelly-Borges et al., 1994; Gunasekera et al., 1994). Identification of microbial associates in *Discodermia* could lead to a better understanding of the ecological or physiological role of these compounds. In this study, we provide a preliminary description of eubacterial species diversity associated with species of *Discodermia*, using both microbiological and molecular tools. A primary goal of this exercise was the identification and matching of *Discodermia*–associated microbe sequences to the closest rRNA relative in current sequence databases. Potentially uncultivable microorganisms were characterised using the polymerase chain reaction (PCR), cloning and DNA sequencing of cloned 16S-like small subunit (SSU) rRNA gene segments (Pace et al., 1986; Lane et al., 1991; Delong, 1998).

Molecular phylogenetic analyses of these sequences highlight the diversity of lineages found within a single sponge genus. Final systematic resolution of each isolate based on rRNA sequence data alone is not attempted in this study.

MATERIALS AND METHODS

COLLECTION AND ISOLATION OF MICROORGANISMS FROM MARINE MACROORGANISMS. Specimens belonging to *Discodermia* spp. used as sources of microbial isolates characterised in this study are listed in Table 1. Isolates from several non-*Discodermia* sponges (*Dercitus*, *Halichondria* and *Corticium* spp.) were included for a cursory comparison with microbe profiles of *Discodermia* spp. (Table 2). All specimens were obtained in accordance with the permits and rules granted by the cooperating sovereign governments. Marine macroorganisms were collected either by SCUBA or through the use of the 'Johnson- Sea-Link' manned submersibles. On return to the surface, sponge samples used for microbial isolation were immediately sub-sampled and prepared for plating using the following method. The sponge was surface-sterilised by rinsing with 70% (v/v) ethanol. A cube of mesohyl (approx. $1cm^3$) was removed using aseptic technique and placed in 20ml sterile artificial sea water (ASW). The sample was then ground in a Waring blender pre-sterilised with 70% (v/v) ethanol, and the resulting suspension was serially diluted with sterile ASW. The dilution series was used as the inoculum for a series of isolation plates. Typically, 100µl of the 10^0, 10^{-3} and 10^{-5} dilutions were used since these were found to bracket the range at which discrete colonies were found.

Sponge cell dissociation and selective cell enrichment were performed by dissociating fresh sponge sections in calcium- and magnesium-free artificial seawater (CMF) (Pomponi & Willoughby, 1994) in a Virtis grinder for 10secs. The resulting slurry was filtered through a 70µm strainer and cells were checked for viability. Cell suspensions were then placed into 15ml tubes and centrifuged to obtain enriched fractions of sponge cells, unicellular bacteria and filamentous bacteria.

Several microbial isolates (e.g. K200, K202, K261) were obtained by fractionating dissociated sponge mesohyl through Percoll/CMF Gradients. A 10ml working solution of 15% Percoll/CMF was diluted with 5M Tris [pH 8.0] from a 90% Percoll/CMF stock solution, and placed in 15ml centrifuge tubes at -75°C for 30mins. About 1ml of sponge mesohyl (5–10g ground in 20ml of filtered sea water) was layered on top of the thawed Percoll/CMF working solutions and centrifuged at 2,000 RPM for 20mins. Tubes were punctured approximately 2.5cm from the bottom, and five 2ml fractions were collected into 24 well plates. About 100µl of each fraction was then plated onto the appropriate isolation media (see below).

Isolation media used in this study were: 1) Chitin Sea Water: Colloidal chitin (in approx. 25ml deionised H_2O), 0.25g dry weight/liter medium; agar, 20g; ASW, 975ml. 2) LN: Bacto Peptone (Difco), 0.5g; Yeast Extract (Difco), 0.5g; agar, 16g; 75% (v/v) ASW, 1L. 3) M3: K_2HPO_4, 0.466g; NaH_2PO_4, 0.732g; KNO_3, 0.1g; $MgSO_4.7H_20$, 0.1g; Na propionate, 0.2g; NaCl, 0.29g; $CaCO_3$, 20mg; $FeSO_4$, 200mg; $ZnSO_4$, 180mg; $MnSO_4$, 20mg; agar, 18g in 1L deionised H_2O. Cycloheximide (50mg) and thiamine (4mg) were added after autoclaving. 4) ISP2: Difco ISP2 prepared with 75% (v/v) ASW. 5) HSV (humic acid, sodium salt), 1g; glycerol phosphate, 110mg; 75% (v/v) ASW, 1L; 10ml BME Vitamin Mix (Sigma) added after autoclaving.

Inoculated plates were incubated at ambient temperature (approx. 25°C) for 2-4 weeks. After this period of incubation, discrete colonies were transferred to fresh plates of the isolation medium, incubated and then re-streaked until the isolate was axenic. Isolates were then transferred to Marine Agar 2216 (Difco). All strains used in this study were maintained as Marine Agar 2216 slant cultures.

DNA ANALYSIS. Genomic DNA from bacterial isolates and sponge mesohyl fractions was extracted using standard purification methods (Sambrook et al., 1989), although some of this DNA required alternative purification methods or modifications which have been previously described (Pitcher et al., 1989). Segments of the 16S-like small subunit (SSU) nuclear rRNA gene amplified from bacterial cultures were sequenced directly after purification of the PCR product. Products derived from sponge mesohyl fractions were cloned before sequencing based on the following procedure.

The universal eubacterial primers, Ecoli9 [5' GAG TTT GAT CAT GGC TCAG 3'] and Loop27rc [5' GAC TAC CAG GGT ATC TAA TC 3'], amplify about 800bp of the 5' end of SSU rRNA gene under standard PCR conditions (Lane, 1991). The segments encompass variable

TABLE 1. Profile of *Discodermia* samples used for microbial characterisation. Key: 1, sample was obtained at a similar location and depth as for the samples of species not belonging to *Discodermia* (as indicated in Table 2). 2, *Discodermia* sample 20-XI-97-1-001 is listed twice because it was used to both a) isolate 51 microorganisms of which two have been analysed, and b) to derive the eight PCR-amplified products comprising the 28 and 29 clone series, which were not cultured.

Discodermia sponge sample ID	Location collected	Depth (ft.)	Total no. microbial isolates	No. sequences analysed
21-III-87-3-014	Bahamas	100	5	2
18-III-87-3-001	Bahamas	515	2	2
8-XI-90-1-001	Bahamas	592	9	2
1-XII-92-2-001	Bahamas	110	13	1
7-XII-92-2-001 [1]	Bahamas	540	8	1
17-XII-92-1-005	Bahamas	535	10	1
15-I-96-2-012	Bahamas	520	1	1
27-X-96-1-003	Bahamas	543	59	7
29-X-96-4-006	Bahamas	520	8	1
9-XI-97-3-008	Honduras	383	65	2
16-XI-97-1-004	Honduras	440	100	2
20-XI-97-1-001 [2]	Honduras	415	51	2
20-XI-97-1-001 [2]	Honduras	415		8
Totals			331	32

regions V1–V4 of bacterial SSU rRNA (*E. coli* positions 9–804) and were expected to provide sufficient genetic variation for phylogenetic analyses (Lane, 1991; Liesack et al., 1991). This size also facilitated complete sequencing of both strands with a minimal number of sequencing reactions, using the above amplification primers and internal primers int-250f [5' GAC TCC TAC GGG AGG CAG 3'] and int-275rc [5' CAC GCG GCG TCG CTG CAT 3']. The typical PCR amplification profile was 94°C denaturation for 45secs, 53-55°C annealing for 60secs, and 72°C extension for 60secs, repeated for 30 cycles. PCR products conforming to expected molecular weights were purified by gel isolation or Qiagen columns (Qiagen), and sequenced by the dye-terminator cycle sequencing method (Applied Biosystems Inc - ABI) run on ABI 373 automated DNA sequencers (University of Florida, ICBR, DNA Sequencing Core Lab, Gainesville FL). To identify potentially uncultivable microbes, PCR products derived from either total mesohyl preparations or enriched mesohyl fractions were 'shotgun' cloned into TA vectors according to manufacturers' instructions (Invitrogen). Clones derived in this manner were designated a number beginning with either 28 (enriched fraction) or 29 (total mesohyl preparation).

All rRNA gene sequences were analysed with the GCG DNA analysis package (GCG, 1994) and SEQED data editor (ABI). The most conserved rRNA sequences relative to *Discodermia*- derived microbes were identified using queries generated by SIMILARITY-RANK in the Ribosomal Database Project (Maidak et al., 1994), or by BLAST using GenBank (Altschul et al., 1990). Preliminary alignments of sequences were made using PILEUP (GCG, 1994), followed by a manual verification for the presence of canonical rRNA secondary structures and compensatory base changes (Neefs et al., 1993; Gutell et al., 1994) (also see Fig. 1). A gap extension penalty of 1, rather than the default of 4, maximised similarity by allowing longer gaps. Maximum parsimony analysis was performed with PAUP, version 3.1.1, while neighbour-joining (NJ) and maximum likelihood (ML or DNAML) analyses were performed with PHYLIP 3.572 software, (Felsenstein, 1993; Swofford, 1993; Hillis et al., 1996). Bootstrap replications of datasets were performed a minimum of 100 times and individual taxa (or operational taxonomic units, or OTUs) which appeared to be problematic (exhibiting long branch lengths or many uninformative nucleotide substitutions) were jackknifed (Efron, 1982). Typical heuristic searches in PAUP utilised evaluations of at least 50 replications of random sequence additions, tree bisection-reconnection (TBR), and decay index assessments (Hillis et al., 1996). Subsets of the total dataset of over 40 OTU's (including

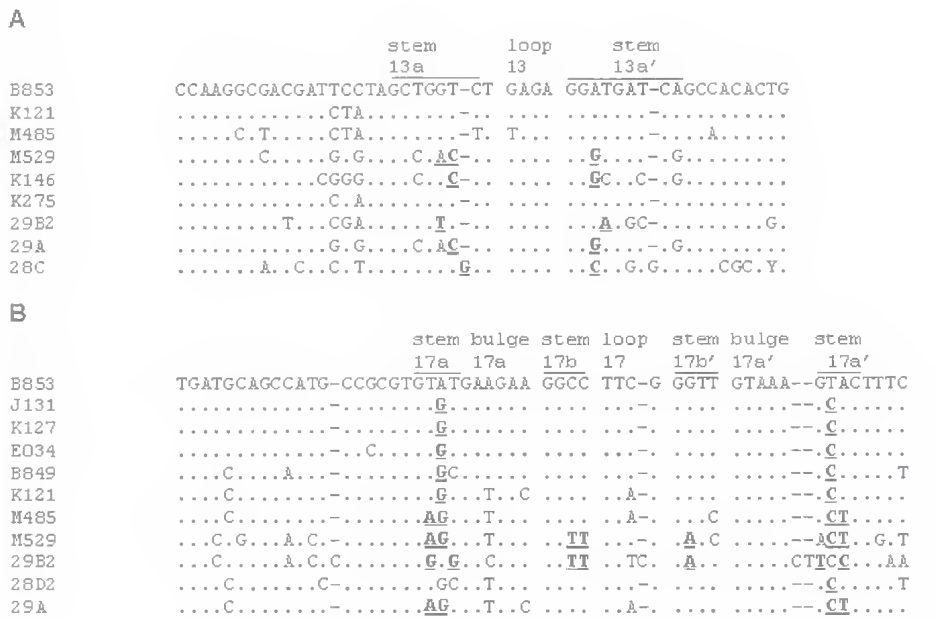

FIG. 1. Representative partial alignments of SSU rRNA regions with conserved secondary structures (Neefs et al., 1993; Gutell et al., 1994). Structures corresponding to: A, Stem/Loop 13; B, Stem /Loop and Bulge 17 are shown over the nucleotide sequences. Both strands of a stem are underlined with the downstream structure marked by a (') above the sequences. Stem 13 in (A) and stem 17 (B) begin at *E. coli* position 288 and 405, respectively. Stem 13a' contains an asymmetric bulge, lengthening it relative to upstream 13a. Nucleotides identical to the first reference sequence are shown below as dots (.); gaps are indicated by (-); compensatory mutations in all stems are underlined.

outgroups listed below) were analysed to verify support for each clade. For NJ, genetic distances were calculated with DNADIST, also in PHYLIP, using Kimura's 2N parameter correction for multiple substitutions and empirically derived transition/transversion ratio of 2.0. Although each maximum likelihood analysis was limited to a subset of 20 OTUs, different taxa within major clades were interchanged and substituted in separate runs of the program to monitor consistency of the consensus topology. NJ and DNAML analyses were performed on a Digital AlphaServer 8400 mainframe computer maintained at the Frederick Biomedical Supercomputer Center in Frederick, Maryland.

Chimeric sequences were detected by comparing the identity of 5' and 3' ends separately before making contiguous constructs, or by using the CHECK-CHIMERA option in RDP (Liesack et al., 1991; Maidak et al., 1994; Rheims et al., 1996). When chimeric products were found, each respective terminal sequence was analysed as a single entity up to the artificial crossover junction and included in phylogenetic analyses despite the shorter length of rRNA sequence.

Sequences (with GenBank accession numbers) of the following representative eubacterial strains and genera were used as outgroups or as reference sequences for the major clades observed in phylogenetic reconstructions: *Bacillus firmus* (X60616), *Pseudomonas straminea* (D84023), *Capnocytophaga* sp. (X97245), *Alteromonas macleodii* (X82145), *Vibrio alginolyticus* (X74690), *Ridgeia piscesae* (U77480), *Thermotoga maritima* (M21774), *Actinomycetes* spp. (X92705), *Rhodobium marinum* (M27534), *Burkholderia solanacearum* (U28232), *Clostridium* sp. (L09175, X77837), *Chloroflexus* (D38365), *Lyngbya* (AJ000714) and an unidentified marinobacter (U61848).

RESULTS

MICROBIAL ISOLATIONS. Phenotypic identifications of microbial isolates were made by analyses of colony morphology, microscopic observation and Gram staining. A general

TABLE 2. Comparison of isolates from different sponge species collected at similar depths, locations and times as for samples of *Discodermia* (listed in Table 1). The number given for *Discodermia* is the average for the three samples analysed.

Sponge taxon	No. of sponge samples used for analysis	No. of Isolates		
		Gram-positive bacteria	Gram-negative bacteria	Fungi
Discodermia	3	5	3	1.5
Dercitus	1	4	4	
Corticium	1	8	1	
Halichondria	1	3	1	3

taxonomic grouping of microbial isolates was obtained from different sponge taxa proximal to, or at similar location, depth and time as *Discodermia* samples (Tables 2, 3). Although major microbial groups such as eubacteria, actinomycetes and fungi were identified, archaebacteria and protists were not cultured. Fungal isolates and their sequences will not be discussed in this paper. To investigate any possible trends or biases in microbial isolations, the number of isolates in different microbial categories obtained from different sponge taxa and different subsamples are summarised in Table 3. These values indicate that there are different profiles of microbial populations among different species of sponges, and that there is also potential variation in microbial yields among different samples of a particular sponge species. For example, some variation in isolate yields appeared to be related to geographical differences in sample collection, whereas specimens of particular species from different depths did not exhibit any strong trends. Some variation may also be attributed to changes in the criteria used to select colonies for isolation and in the media used for certain experiments.

EUBACTERIAL STRAIN IDENTIFICATION. The names of eubacterial strains resulting from highest identity scores are listed in Table 4, together with their corresponding percentage sequence identities. Relatively good agreement was observed between the two major sequence datasources, RDP and GenBank databases, with novel bacterial rRNA sequences from cultured isolates used to infer possible taxonomic placements. However, several caveats to this procedure should be emphasised. 1) Database searches revealed only the most similar sequence in the respective database, and these were not considered as an absolute identification of a particular 'species' or strain of bacteria, even when sequence identity exceeded 99% (Fox et al., 1992). Indeed, it is probably more appropriate to make reference to a specific 'rRNA type' or strain than to infer that these identifications are homologous to species-level taxonomy. 2) Since it was not possible to obtain multiple operon sequences or samples of any given isolate, it was therefore not possible to assess possible intra-strain or intraspecific variation amongst the microbial taxa (Clayton et al., 1995).

The wide taxonomic diversity observed in this survey was striking. For example, at least three major eubacterial divisions, or five classes (Woese, 1987; Balows et al., 1992), are represented in the microbial isolates obtained from samples of *Discodermia*: the gamma-, beta-, alpha- proteobacteria, cytophaga, and Gram-positive eubacteria. Of the 24 isolate sequences obtained from *Discodermia* the most commonly observed bacterial subdivisions were gamma-proteobacteria (9) and alpha-proteobacteria (8), followed by Gram -positive (4), beta-proteobacteria (2) and possibly a single *Cytophaga*-like isolate (K279) (Table 4). This Gram-negative isolate matched most closely a psychrophilic marine *Cytophaga*, with some regions reaching 93% correspondence in identity, although these sequences were still not fully confirmed at the time of writing. Nonetheless, detection of *Cytophaga* primarily from the surfaces of marine aggregate particles in marine systems has been previously described (Delong, 1998).

There were only a few cases where sequence database matches appeared unequivocal: e.g. K169 showed high similarity to *Pseudomonas stutzeri*, a common Gram-negative microbe in RNA group 1 (Balows et al., 1992), and E034 appeared to be related to a bacterium first characterised from Pele's hydrothermal vents in the Pacific ocean. In other instances *Discodermia* isolates matched sequence database entries at highly significant identity levels (97-100% similarity), but these matches were made to 'anonymous' strains identified only to the genus or even subdivision level. For example, the closest relatives to isolates K171 and J131 were an 'alpha-proteobacterium MBIC3368' and *Alteromonas* sp., respectively. Moreover, comparison of these two isolates maintained high sequence conservation across the whole rRNA segment, in contrast to 28D which had a range of conservation values (77-93%) largely dependent on the region of the gene under comparison (Table 4). Since several species of *Vibrio* exhibited equally

TABLE 3. Profile of isolates from different sponge species collected at depths and habitats similar to *Discodermia*. Bold face type indicates samples that were obtained at similar locations or on the same expedition, and thus reliable for more direct comparisons between samples.

Sponge taxon	Number of samples used for isolation	Depth (ft.)	Collection location	Bacteria		Actinomycetes	Fungi
				Gram Pos.	Gram Neg.		
Halichondria	21	390	W. Barbados				1
		526	Canary Islands				1
		477	Bahamas	2	4		
		393	Bahamas	4	1	1	2
		1386	Bahamas	5		1	
		750	Bahamas	1	1	1	1
		1803	Bahamas	5	1		1
		803	Bahamas	2	1		1
		440	Bahamas	1	1		1
		576	Bahamas				4
		543	**Bahamas**	**3**	**1**		**3**
		473	Bahamas		1		
		479	Jamaica	4	9		1
		520	Jamaica		3		2
		1497	Jamaica				1
		410	Jamaica				1
		480	Bahamas	6			3
		230	Bahamas	1			
		149	Florida, east coast	12	33	3	5
		472	Puerto Rico	3	73		2
		450	Puerto Rico		1		
Dercitus	10	20	Venezuela			2	3
		455	Bahamas			1	4
		504	Bahamas				
		540	Bahamas			1	1
		432	Bahamas	6	1	1	
		700	Bahamas	6	3		1
		525	**Bahamas**	**4**	**4**		
		806	Jamaica	3		1	
		400	Jamaica	15	1		3
		384	US Virgin Islands		3		
Corticium	9	581	Bahamas			1	
		525	**Bahamas**	**8**	**1**		
		462	Bahamas	1	2		1
		443	Bahamas	4	8		1
		305	Bahamas		2		
		2819	Bahamas	1	3	1	
		377	Bahamas	8			1
		1585	Turks & Caicos		1		
		90	Puerto Rico	5	11	2	3
Discodermia	10	592	Bahamas				1
		110	Bahamas	7	5		1
		540	**Bahamas**	**4**	**3**		**1**
		535	Bahamas	3	2		5
		520	Bahamas	8	5		1
		543	Bahamas	21	31	5	2
		520	Bahamas	3	2		3
		383	Honduras	9	51	1	4
		440	Honduras	36	61	1	2
		415	Honduras	14	25		12

high scores in comparison to the isolate K261 (ca. 98%), only the genus is listed in Table 4.

Although we did not deliberately attempt to detect or amplify cyanobacteria, this group is well known amongst sponges living in the photic zone (Wilkinson, 1987; Ruetzler, 1990; Diaz, 1997), and some of our samples of *Discodermia* were collected in or near this zone. Clone 28C exhibited strong sequence similarity to a *Leucothrix*, which has been described as a large-diameter, morphologically distinct, marine gliding bacteria related to cyanobacteria (Balows et al., 1992).

To facilitate direct comparisons between our sample isolates and known sequences, and to monitor sampling variation, we undertook parallel rRNA sequence analysis of potentially different microbial isolates from other sponge taxa living in geographical proximity to *Discodermia* (Table 2). These isolates (indicated in boldface in Table 3) were derived from sponge samples collected from the same habitats and depths as *Discodermia* (indicated in bold in Table 1). These few data, although preliminary, suggest a higher frequency of a *Bacillus* in non- *Discodermia* sponges, which is possible circumstantial evidence for sponge-specific microbial associations (Althoff et al., 1997) (see Table 3). More extensive comparisons could be designed to determine the optimal and natural conditions of *Discodermia*-associated microbes, perhaps by wider sampling of proximal sponge and non-sponge habitats (e.g. sediments, seawater, etc.).

PCR was also used to directly amplify rRNA sequenees from a) a total sponge cell preparation, and b) a dissociated mesohyl fraction enriched for specific populations of microorganisms. Although only a small number of rRNA clones were obtained by shotgun cloning from these two sources (labelled 28A–Z for the enriched fraction and 29A–Z for the total 'crude' mesohyl preparation), their identities and overall compositions appeared to be different from those derived from cultured isolates (Table 4). In spite of the fact that these sequences also exhibited the highest frequency of chimeric PCR artifacts (Liesack et al., 1991), precluding analysis of the total rRNA fragment, some of these clones may represent 'uncultivable' microbes. Up to 99% of naturally occurring microbes may be overlooked by standard culturing techniques (Button et al., 1993; Amann et al., 1995; Hulgenhotz & Paee, 1996). For example the 5' ends of two clones in the 29 series appeared to significantly match *Flectobacillus*, along with some conservation (82-91%) to *Ridgea* and *Riftia* hydrothermal vent bacteria (Feldman et al., 1997). Sequences of other *Discodermia* isolates exhibited significant similarity to bacteria associated with hydrothermal vent habitats and organisms. Also, clone 29B2 appeared to be distantly related to *Clostridia*. Furthermore, many mierobes have not yet been analysed or may not have been isolated from the original plates. Interestingly, the sequences of most of the PCR-derived rRNA clones exhibited identity levels below 90%, and thus it may be recommended that unidentified strains with this level of conservation to any entry in either database be considered strong candidates for 'novel' species designation.

PHYLOGENY OF NOVEL ISOLATES. Phylogenies constructed from the rRNA sequences were used to characterise the diversity and relatedness of *Discodermia* bacterial strains. Figures 2 and 3 display dendrograms constructed with two different phylogenetic algorithms: maximum parsimony and neighbour-joining (NJ), respeetively. Maximum likelihood (ML) analyses were also performed with smaller subsets of taxa. Relationships constructed under the principal of parsimony use a criterion of minimum evolution (shortest tree), while NJ uses a clustering algorithm based on overall similarity or distance in a comprehensive OTU×OTU matrix of corrected pairwise distances (Hillis et al., 1996). Although NJ and ML reconstructions better compensate for rate variation among different lineages (Hasegawa & Fujiwara, 1993), ML analyses involved fewer taxa due to computational limitations for large datasets, and thus are not discussed further in this work.

Despite relatively large differences in rRNA sequences among some of the taxa, sequenec alignments appeared to be robust. Multiple invariant positions and highly conserved regions corresponding to previously described secondary structures (e.g. loop 20, loop 14-15, and stem 5) were observed by eye along the nearly 800bp of rRNA sequences. Observation of compensatory mutations in stem 13 and bulge 17, among others, in the novel bacterial rRNAs corroborate the conservation of those structures (Fig. 1). Only one highly variable region corresponding to loop 11 (Neefs et al., 1993; Gutell et al., 1994) required removal due to ambiguous alignment and its effect on nucleotide site homology.

For the 30 microbial taxa analysed by maximum parsimony (Fig. 2), an estimation of

TABLE 4. *Discodermia*-associated microbes identified by 16S-like rRNA sequences. Key: 1, the list of organisms was derived directly from the output of GenBank or RDP queries (Altshchul et al., 1990; Maidak et al., 1994). The names of the closest relative may refer to species, genus or common names. 2, percent identity was derived from BLAST scores only, and may reflect identities of different segments of a single query rRNA sequence. Thus, variable conservation of different regions of the rRNA sequence is indicated in the ranges of identity values shown. 3, since these clones were shown to be chimeric, whole contigs were not analysed and query results reflect identities for 5' termini only. 4, five microbial sequences derived from non-*Discodermia* sponge microbes are underlined - M234 and M196 (*Corticium*), M162 and M099 (*Halichondria*) and M119 (*Dercitus*). 5, clonal sequences indicated with an asterisk (*) represent the 28/29 clonal series which was derived from PCR amplification of sponge mesohyl fractions. Therefore, these sequences were not derived from cultured isolates.

Most closely related genus or group[1]	Microbial ID no.	% Sequence identity[2]
ALPHA		
Alpha proteobacteria	K200	90
Alpha proteobacteria MBIC3368	K202	97
Alpha proteobacteria	K121	88
Alpha proteobacteria	K126	91
Alpha proteobacteria	K275	88-96
Alpha proteobacteria	M485	94
Alpha proteobacteria	M162[4]	97
Erythrobacter	E035	98
Alpha proteobacteria MBIC3368	K171	99
Phodospirillum	29A*	90
GAMMA		
Hydrothermal vent bacterium	M119[4]	97
Vibrio	B853	96
Alteromonas	J131	100
Hydrothermal vent bacterium	E034	99
Vibrio	K261	98
Pseudoaltermonas	K127	97
Vibrio alginolyticus	K141	97
Microbulbifer	C724	89
Unidentified gamma	C723	91
Pseudomonas stutzeri	K169	99
Leucothrix mucor	28C[3] *	80-94

Most closely related genus or group[1]	Microbial ID no.	% Sequence identity[2]
BETA		
Unidentified marine proteobacterium	K255	92–96
Alcaligenes	28D2*	85–94
Beta proteobacteria	B849	92–99
GRAM-positive		
Bacillus (low GC)	M680	96
Bacillus (low GC)	M529	97
Bacillus firmus	M099[4]	91
Bacillus fusiformes	28X*	91
Bacilllus firmus	M196[4]	97
Bacillus firmus	M234[4]	91
Unknown actinomycete	28D*	91
Nocardia, actinomycete	K146	97
Nocardia, actinomycete	K145	80
CYTOPHAGA		
Marine psychrophile	K279	94
AMBIGUOUS GROUPING		
Flectobacillus	29W*	85
Flectobacillus	29B[3]	82–91
Clostridia	29B2*	76–81

skewness of tree length distributions (i.e. for 10,000 random trees using the Random Trees option in PAUP), yielded a g_1 statistic of -0.64. This value is above the 99% significance level for the corresponding critical value of g_1 for more than 25 taxa, indicating a strong leftward skewness and high phylogenetic signal in a four-state character dataset (Hillis & Huelsenbeck, 1992). Weighting transversions over transitions by a factor of 2 shortened the overall length of MP trees. There were only two and four more trees that were one or two steps longer, respectively, than the shortest tree shown in Figure 2 using the same dataset. The clade containing beta and gamma bacteria shows the weakest support (55%), and is thus depicted as a polytomy. Low support is likely due to the uncertain placement of clones 29B, 29W and 28C. Proximal *Ridgeia* and *Marinobacter* groups are typically grouped with gamma proteobacteria.

In parallel, neighbour-joining analyses of rRNA sequence data yielded very similar conclusions to parsimony (Fig. 3). Pairwise genetic distances of all OTU's based on Kimura's 2N parameter correction (Hillis et al., 1996), ranged from 0.04->0.70. The major differences between the NJ and MP trees were: 1) higher bootstrap support for individual clades with NJ relative to MP; 2) fewer collapsed nodes and polytomies with NJ, providing clearer groupings of major proteobacteria subdivisions; 3) inclusion of *Rhodobium* and clone 29A with the cluster of Alpha eubacteria;

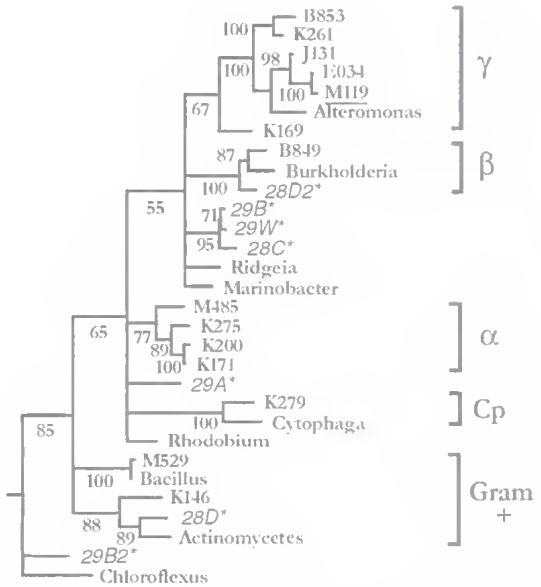

FIG. 2. Representative maximum parsimony phylogeny of *Discodermia*-associated microbes. Cultured isolates are shown in standard bold font. PCR-derived/uncultivated clones are shown with asterisks (*) and in italics, while non-*Discodermia* microbe M119 is underlined. Numbers below each node refer to the bootstrap value after 500 iterations. Representatives of genera or families are listed in the Methods and have names written out. Preliminary heuristic searches using the same dataset, 10 random stepwise additions of taxa with tree bisection reconnection (TBR), found only 2 most parsimonious trees. Length of the two most parsimonious heuristic trees was 1864 steps, with a consistency index (CI) of 0.495. Sub-optimal trees that were longer by 1 or 2 steps numbered only 2 and 4, respectively, and retained the same basic topology of the bootstrap consensus. Morever, the heuristic trees did not collapse the beta and gamma proteobacteria clades into a single polytomy, but rather showed the beta proteobacteria as a distinct group relative to the gamma bacteria (McDonald et al., 1997). Preliminary groupings in the proteobacteria subdivisions and *Cytophaga* (CP) were based on the identities obtained from BLAST sequence database searches.

and 4) monophyly of all representative Gram-positive bacteria. Phylogenetic assignment of clone 29A, which appeared most closely related to the alpha subgroup, was problematic with all three algorithms (ML tree not shown). The decay index for any group that included 29A was always low (<3). Since it is not within the scope of this study to evaluate the strengths and weaknesses of various phylogenetic methods, or to make definitive conclusions on the taxonomic status of each novel isolate, some of these taxonomic placements are likely to be revised in the future.

Nevertheless, the topologies of parsimony and distance trees were generally consistent in showing at least 5 major clades. Isolates K261 and K171 were at the tips of the MP tree and thus their omission to accelerate computation times did not have a significant effect on NJ tree topology. Several features in the present reconstructions of *Discodermia* microbes, such as the monophyly of all proteobacteria, strong bootstrap support for the gamma and beta subdivisions, and the outgroup status of the Gram-positive clade, are all in agreement with current bacterial taxonomy. More specifically, a long branch characterised the lineage of K279 and its strong association with *Cytophaga* bacteria. This branch is considered by us to be a fifth major bacterial clade of the sponge. Relatively long branch lengths were prominent for several other lineages (e.g. K146 and some clones in the 28/29 series).

Although uncultivated clones 28C, 29B and 29W were grouped significantly with other marine bacteria discovered from previous environmental surveys (Moyer et al., 1995; Fuhrman & Davis, 1997), the database matching of 29W and 29B to *Flectobacillus* (a Cytophagales) indicates that accurate placement of these bacteria require more refined determination. However, the tight clustering of taxa observed also suggests possible endemism or ecological specificity with respect to *Discodermia*. Consistent with the database matches to *Clostridia*, clone 29B2 was placed repeatedly near outgroup taxa at the base of all trees. The weak and unresolved positions of some taxa, such as 29B2, 29A and K146, connote another level of diversity. The inclusion of non-*Discodermia* isolates (M119, M169, M234, M162 or M099) in some reconstructions did not significantly alter tree topologies, nor did it suggest any evidence of taxon-specific symbioses occurring in the current dataset. Overall, these results parallel earlier descriptions (Santavy et al., 1990) describing major bacterial groups such as *Vibrio*, *Aeromonas*, and coryneform/actinomycete (Gram-positive) strains derived from marine sponges.

DISCUSSION

Phenotypic, comparative DNA sequence and molecular phylogenetic analyses confirmed the presence of at least five distinct eubacterial clades of 16S-like SSU rRNA sequences from microbial

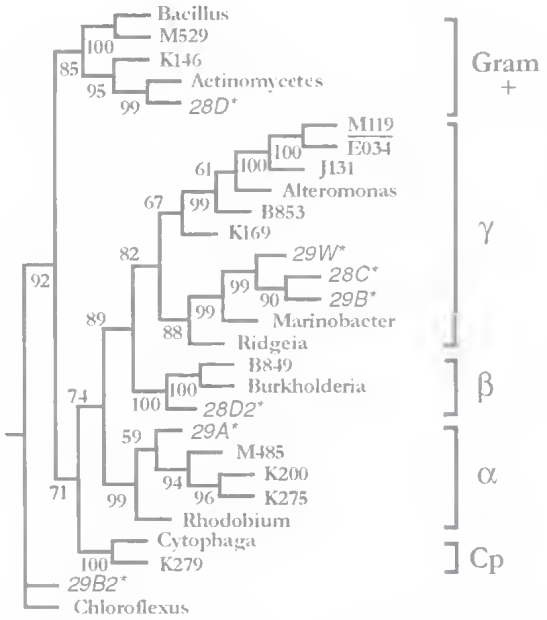

FIG. 3. Distance-based phylogeny reconstructed with the Neighbour-joining method. The same taxa (except K171 and K261) and annotations as that shown in Fig. 2 were analysed.

isolates of the lithistid sponge genus *Discodermia*. Several of the identified *Discodermia* bacterial groups, such as the gamma proteobacteria, are consistent with previous characterisations of deep-sea microbes (Moyer et al., 1995; Feldman et al., 1997; Fuhrman & Davis, 1997), while other lineages (e.g. K279, 28C, 29W, 29A) appear unallied or novel. This situation may have arisen via accelerated substitution rates, while group- or strain-specific synapomorphies were maintained (Hillis et al., 1996; Peek et al., 1998). More likely, however, no close relatives exist in current prokaryotic rRNA sequence databases, which underscores possible missing links in current bacterial rRNA taxonomies. Similar to many earlier surveys of bacterial diversity from the environment (Pace et al., 1986; Giovannoni et al., 1990; Amann et al., 1995; Rheims et al., 1996), PCR and molecular methods have probably revealed unique microorganisms which are otherwise uncultivable under traditional methods. Although some variation may be attributed to differences among species of *Discodermia* and between individual samples of particular species, this characterisation also likely underestimates total diversity in the genus, since only about 10% of the *Discodermia* isolate collection has been sequenced at the time of writing. Nevertheless, our finding that many different bacterial strains and rRNA 'types' stem from only one sponge genus is novel and distinguishes the present study from earlier results.

Bacterial symbionts occur both intracellularly and extracellularly with respect to their sponge host mesohyl (Vacelet & Donaday, 1977; Simpson, 1984; Wilkinson, 1987). However, without positive identification of the types of interacting organisms, elucidating symbiotic parameters such as nutrient transfer, detoxification or gene exchange will not be as meaningful as those made for well-established cnidarian-dinoflagellate associations (Trench, 1993). It is possible that some of the microbes identified here stem fortuitously from the microbial pool derived from sponge filter-feeding activities (Reiswig, 1971; Pile et al., 1996). A long-standing question in sponge-microbial symbioses has been how do so many different symbionts coexist and seemingly thrive in the relatively inhospitable (phagocyte-filled) environment of the marine sponge mesohyl (Simpson, 1984; Wilkinson, 1987)? One answer may stem from the advantages of 'ecto-symbioses' and bacterial communities (Bull & Slater, 1982).

Conversely, it is not unreasonable to suppose that a fraction of the bacterial species isolated and characterised here represent bona fide obligate symbionts of *Discodermia*, an expectation which has been confirmed in other sponges (Burlando et al., 1988; Althoff et al., 1998). Although not trivial, the question of determining specific microbial symbionts could be approached by probing for consistent rRNA (or other genetic) signatures among a matrix of geographically separated samples of *Discodermia* present in our collection. Definitive conclusions on the relative abundance of a particular bacterial strain in *Discodermia* are precluded, since the quantitative recovery of several microbial types during sequencing may suggest any of the following: 1) a dominant presence in the host sponge and concomitant functional role in *Discodermia* physiology; 2) habitat-specific differences; or 3) experimental bias of PCR primer binding sites, genomic DNA quality, etc. (Rheims et al., 1996). It would be interesting in the future to determine whether the mode of molecular evolution in these microbes matches other observations of faster nucleotide substitution rates in symbiotic versus free-living marine bacteria, which may be a function of small population sizes of some symbiotic communities (Peek et al., 1998).

The relatively large breadth and depth of phylogenetic diversity found among *Discodermia*-associated microbes, as revealed by SSU rRNA sequences, has significance in several areas. Firstly, the data reiterate previous studies showing that some marine sponges either maintain or tolerate high levels of microbial species richness. Consequently, this study supports claims that current numbers of catalogued bacterial species are underestimated (UNEP, 1995; Hawksworth & Colwell, 1992; Colwell, 1997). Moreover, the low frequency of duplicate rRNA sequences observed in this survey supports the large diversity of microbes in some sponge taxa.

Lastly, these results may have ramifications for microbial and deep water ecology. Since viable deep sea habitats are generally 'patchy' (Grassle, 1991; Cavanaugh, 1994; Snelgrove & Grassle, 1995), sponges such as *Discodermia* may represent essential stepping stones for bacterial dispersal across large expanses of the seafloor bottom. Such functions are often attributed to less common and more random sinking detritus, animal carcasses (e.g. whale falls), or hydrothermal vent habitats (Showstack, 1998; Tunnicliffe & Fowler, 1998). At the level of the organism sponges may embody oases of species richness, rather than oases of biomass, which is the perception often associated with hydrothermal vents (Snelgrove & Grassle, 1995). The detection of hydrothermal vent-like microorganisms in *Discodermia*, regardless of whether or not they are actual sponge symbionts, suggests a possible source of colonisers for deep water habitats. Thus, *Discodermia* and similar marine sponges should be re-evaluated in the context of a potentially pivotal role in marine microbial ecology, dispersal, and evolution.

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